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FINAL REPORT

IDENTIFICATION OF BACTERIAL FLORA IN GALLERIES OF THE SOUTHERN PINE BEETLE, *DENDROCTONUS FRONTALIS* ZIMMERMAN

(Cooperative Agreement #19-178 with LSU at Alexandria)

By

James E. Marler
Assistant Professor
Dept. of Science-Microbiology
LSU at Alexandria

Stanley J. Barras
FIDR Staff, USDA
Rosslyn, VA

RWU 2203

Southern Forest Experiment Station

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Final Report Summary
James E. Marler - Alexandria, LA and
Stanley J. Barras - Washington, D. C.

IDENTIFICATION OF BACTERIAL FLORA IN GALLERIES OF THE SOUTHERN PINE BEETLE, *DENDROCTONUS FRONTALIS* ZIMMERMAN

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In a study of the galleries of the SPB 520 culture isolates were made over a 5-month sampling period (June, July, November, December, January, 1975). The following genera were found: *Aeromonas*, *Achromobacter*, *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Enterobacter*, *Flavobacterium*, *Micrococcus*, *Proteus*, *Pseudomonas*, *Serretia* and *Staphyloccus*. Twenty different species or strains were also recorded from these (12) genera. A statistical analysis of species variance by gallery position and month indicated no significant difference. The microbial population appears to be a stable factor in galleries of the SPB.

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Prepared by:

James E. Marler 3/30/78

James E. Marler Date
Assistant Professor
Dept. of Science-Microbiology
LSU at Alexandria

Stanley J. Barras 3/28/78

Stanley J. Barras [1] Date
Principal Entomologist
RWU 2203

Approved:

Peter L. Lorio, Jr. 5/10/78

Peter L. Lorio, Jr. Date
Project Leader
RWU 2203

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[1] Dr. Barras is presently assigned to the Forest Insect and Disease Research Staff, USDA, Rosslyn, Va.

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INTRODUCTION

In 1973 we started a study of the microflora of the southern pine beetle (SPB). The first phase dealt with the beetle itself, with isolations from the fore-, mid-, and hindgut portions of the digestive tract (Barras and Marler 1974). Over 300 cultures were obtained, representing 11 genera and 23 possible species of bacteria. Oberle (1966) and Moore (1972a) reported finding 6 genera and 6 to 9 species of bacteria in their studies of the internal flora of the SPB. In our first study, hyphal fungi and yeasts were also isolated and catalogued as to location. They were not identified but most of the yeasts were morphologically similar to the genera *Candida*, *Hansenula*, and *Pichia* which are commonly associated with the SPB.

The present study was to determine the dynamics of the in-gallery bacterial population and to determine the variation in genera number and composition from summer to winter. The results may also give an insight into microbial competition within the galleries.

METHODS

Galleries were sampled in 60 cm bolts of naturally infested loblolly pines. Three trees were selected during June and July, and 3 trees were selected during November, December, and January. Two bolts were taken from each infested tree. Following the removal of the rough outer bark, the bolts were placed in a microbiological transfer chamber and surface sterilized with flaming alcohol. Using sterile knives the bark was removed to expose the entire length of 2 galleries in two bolts from each month. The following parts of the galleries were sampled: 1) head of gallery - region where females were actively constructing egg niches; 2) middle gallery - frass in the egg niche; 3) egg niche - phloem from the walls of the egg niche; 4) larval galleries - phloem from the young larval galleries branching off the adult gallery; 5) Frass - frass from the older end of the galleries.

Samples were aseptically removed from the above locations with flame sterilized forceps or scalpels. In addition, an extra sample was taken from beetle-free phloem to monitor technique and extraneous contamination. A contamination rate of less than 1% indicated that this sampling system was satisfactory.

Samples were placed in a nutrient broth (tryptic soytone broth-Difco) and shaken mechanically to dislodge and disperse the bacteria. All cultures were incubated at 25°C for 3-5 days. The samples were then subcultured onto tryptic soytone agar for isolation.

Results of standard biochemical and cultural tests were used for numerical analysis identification (Sneath and Sokal, 1973), (Table 1). The isolates were assigned a code number and characterized onto computer forms.

RESULTS AND DISCUSSION

In our study of the galleries of the SPB, 520 culture isolations were made over the 5-month sampling period (Table II). The following genera were isolated from the galleries: *Aeromonas, Achromobacter, Alcaligenes, Bacillus, Brevibacterium, Enterobacter, Flavobacterium, Micrococcus, Proteus, Pseudomonas, Serratia,* and *Staphylococcus.* Many of the species previously found in the gut were found in the galleries, but the major difference was the presence of a wider variety of fermentative gram negative rods in the galleries. *Serratia, Proteus, Enterbacter,* and *Aeromonas* were found in many samples throughout the study period. These organisms are opportunistic pathogens in many insects so their presence was not unexpected (Moore 1972b; Stairs 1972, Steinhaus 1959). These organisms may be indicative of deteriorating conditions within the galleries.

In terms of numbers of isolates, the middle galleries and egg niches contained the highest populations, 145 and 126, respectively. The larval galleries and head of galleries were next with 115 and 122 isolates, respectively. The frass packed older end of the galleries contained very few viable organisms, 12 isolates (Table II).

A statistical analysis of the results showed no significant difference in species variation during the 5 month sampling period. The exception was the #5 position, Frass. However, this gallery position had such low numbers of isolates that we conclude that frass does not support the growth of bacteria (Tables V and VI).

The computer program for numerical analysis identification was set up using the 7th edition of Bergey's manual. Before the study was completed the 8th edition of Bergey's manual was published. Many of the species described in the 7th edition were changed or dropped completely. Many of the taxa in the computer were 'species incerta sedis'. Rather than rewrite the computer program, we ran it using the 7th edition taxa. Where possible, we have changed the name to agree with the new taxon, but where there is no new species name, we have used the old nomenclature. Pages 273-275 in the 8th edition of Bergey's manual covers some of the problems encountered in keeping up with the new taxomony.

Table III lists the 12 genera and 16 species isolated from the SPB. The following are notes on each of the genera.

Genus *Alcaligenes:* This genus was completely reworked in the 8th edition, retaining only the single species, *A. faecalis*, from the 7th edition. A total of 36 isolates were found to be *Alcaligenes* and by the 7th edition taxonomy 9 (25%) were identified as *A. recti*, and 27 (75%) were identified as *A. faecalis*. However, by the 8th edition taxonomy, all 36 isolated would be considered *A. faecalis*. We record them as *A. faecalis* in line with current taxonomy.

Genus *Achromobacter:* The family Achromombacteriaceae and the genus *Achromobacter* were dropped as valid names from the 8th edition and all previously described species were assigned to other genera. Most of them went into two genera, *Alcaligenes* and *Acinetobacter.* All the motile, oxidase positive forms were considered *Alcaligenes,* while the nonmotile, oxidase negative forms were placed in *Acinetobacter.* However, Tatum *et al.* (1974) believe that all species that are motile, oxidase positive, and that oxidatively metabolize sugars should retain the name *Achromobacter* to distinguish them from the non-saccharolytic *Alcaligenes* and *Acinetobacter.* Until the International Committee on Nomenclature of Bacteria resolves this controversy, we will place all 76 isolates of *Achromobacter* into *Acinetobacter calcoaceticus.* This is the only species listed for this genus.

Genus *Aeromonas:* *Aeromonas* is often identified as *Escherichia* or *Enterobacter* in the family Enterobacteriaceae because it ferments sugars with the production of acid and gas. However, because it is oxidase positive and has polar flagella, it is not in the family Enterobacteriaceae. There were 14 isolates that had the characteristics of *Aeromonas* and all 14 were classified as *A. hydrophilia.*

Genus *Bacillus:* There were 3 species of *Bacillus* among our isolates. Four isolates were *B. subtilis,* 12 were *B. cereus,* and 1 was identified as *B. megaterium.* Although in nature the *Bacillus* are among the most widespread and numerous and are generally well represented in microflora studies, only 5% of the 327 bacteria were *Bacillus.* This could mean that these 17 bacilli were fortuitous contaminants of the beetle and that conditions within the galleries are not conducive to the growth of *Bacillus.*

Genus *Brevibacterium:* This gram positive, asporogenous rod was seen in 6 isolates but none of them fit the species description programmed into the computer, nor were we able to hand key them with certainty. Therefore, they were tabulated as 2 isolates of *Brevibacterium* sp., strain A, and 4 isolates of *Brevibacterium* sp., strain B. The single linkage dendrogram with seven known species cards failed to link any of the 6 isolates with those species. The 8th edition suggests that the *Brevibacterium* species be transfered to either *Corynebacterium* or *Arthrobacter* but does not indicate to which species they would belong.

Genus *Enterobacter:* This genus, a member of the family Enterobacteriaceae, made up 22% of the bacterial cultures isolated. The isolates fell into two species, *E. aerogenes* (64 isolates) and *E. cloaca* (7 isolates). In the study of the microflora of the SPB gut, only 4% of the isolates were of the genus *Enterobacter* versus 22% found in the gallery population. The distribution within the galleries was equal for all positions (except #5, frass) and showed no significant difference by month or position.

Genus *Flavobacterium:* Several pigmented gram negative rods were isolated and identified as *Flavobacterium*. We had 19 isolates of which 5 were *F. lutescens*, 5 were *F. aquatile*, 7 were *F. ferrugineum*, 2 were not identifiable and are listed as *Flavobacterium* sp.

Genus *Micrococcus:* In our SPB gut study, *Micrococcus* was not isolated, whereas in the gallery study this genus accounted for 3.6% of the isolates. Again it appears that conditions are not very favorable for the growth of this genus in the galleries. Table IV shows that half of the positions sampled did not yield *Micrococcus* isolates. Of the 12 cultures isolated, 5 were identified as *M. luteus*, and the other 7 were *M. varians*.

Genus *Proteus:* Only 4 isolates of this genus were recorded. It was not found in the SPB gut and its low incidence in the galleries probably means that it is not associated with the beetle during development in the tree.

Genus *Pseudomonas:* There were 9 gram negative, polar flagellated rods isolated, but no attempt was made to identify species. However, a single linkage dendrogram using the 80% similarity as a breakpoint for difference indicated that there were at least 6 possible strains. Five similar strains were seen in the SPB gut, so perhaps these gallery pseudomonads are from the gut of the SPB.

Genus *Serratia:* About 16% of the isolates belonged to *Serratia* which was fairly evenly distributed along the gallery and over time. *Serratia* is a known pathogen of insects and may play a role in early death of the young larvae as they hatch. All 52 cultures were found to be *S. marcescens* even though they often failed to show the red pigment often exhibited by the genus. It has been the experience of one of us (Marler) that the red pigment production by *Serratia* is variable and greatly repressed at temperatures above 30°C.

Genus *Staphylococcus:* As with *Micrococcus* this genus was seen in less that 4% of the isolates. Eleven cultures were keyed out to *S. epidermidis*. It would appear that both *Staphylococcus* and *Micrococcus* are not part of the endogenous microbial population of the SPB.

Yeast: Yeasts were commonly found throughout the gallery but there was no attempt to identify them. A random spot check was made of the 190 cultures to determine the variation and only three genera were observed: *Candida*, *Pichia*, and *Hansenula*. These yeasts are commonly associated with the SPB and appear to thrive in the galleries of the beetles.

TABLE I

Cultural and Biochemical Observations for each Isolate from the Galleries of the Southern Pine Beetle.

Cultural Observations

1) Microbial group-bacteria
" " -yeast
" " -hyp. fungi
2) Gram stain
3) Motility
4) Flagella type
5) Cell shape
6) Cell grouping
7) Colony color
8) Spores

Biochemical Observations

9) O/F glucose
10) O/F sucrose
11) O/F lactose
12) O/F mannitol
13) Growth on EMB
14) Growth on SS
15) Pigment on PP/PF agar
16) Methyl Red
17) Vogues-Proskeur
18) Citrate
19) NO_3 reduction
20) Amylase

Biochemical Observations

21) Litmus milk acid
22) " " alk/NR
23) " " reduction
24) " " proteolysis
25) Urease
26) Gelatinase
27) Hydrogen sulfide
28) Indole
29) Catalase
30) Oxidase

TABLE II

Frequency of Isolates of Genera of Micro-organism in the Galleries of the Southern Pine Beetle.

Genera	Gallery Position					
	Head	Middle	Egg Niche	Lar. Gal.	Frass	Total
Achromobacter*	10	27	18	18	3	76
Alcaligenes	12	9	6	9	0	36
Aeromonas	5	0	6	3	0	14
Bacillus	4	8	2	3	0	17
Brevibacterium	1	1	3	1	0	6
Enterobacter	20	14	18	19	0	71
Flavobacterium	8	4	4	3	0	19
Micrococcus	3	4	3	2	0	12
Proteus	1	1	1	1	0	4
Pseudomonas	3	2	1	3	0	9
Serretia	8	19	14	11	0	52
Staphylococcus	0	7	2	2	0	11
Yeast	47	49	47	38	9	190
Hyphal Fungi	0	9	1	2	0	3
Total Isolates	122	145	126	115	12	520
% of Total	23.5	27.9	24.2	22.1	2.3	100

*Achromobacter has been dropped in favor of Acinetobacter in the 8th Edition of Bergey's Manual.

TABLE III

Species of Bacteria Isolated from the Galleries of the Southern Pine Beetle.

SPECIES	NO. OF ISOLATES
Acinetobacter calcoaceticus*	76
Alcaligenes faecalis	36
Aeromonas hydrophilia	14
Bacillus subtilis	4
Bacillus cereus	12
Bacillus megaterium	1
Brevibacterium sp. strain A	2
Brevibacterium sp. strain B	4
Enterobacter aerogenes	64
Enterobacter cloacae	7
Flavobacterium lutescens	5
Flavobacterium aquale	5
Flavobacterium ferrugineum	7
Flavobacterium species	2
Micrococcus luteus	5
Micrococcus varians	7
Proteus rettgeri	4
Pseudomonas species	9
Serretia marcescens	52
Staphylococcus epidermidis	11

*Formerly Achromobacter sps.

TABLE IV

Number of isolates of each genus by month and gallery position.

	June Gallery Position				
Genus	Head	Middle	Egg Niche	Larval Gallery	Frass
Achromobacter*	3	5	4	4	1
Alcaligenes	4	3	0	2	0
Aeromonas	0	0	0	0	0
Bacillus	0	2	0	0	0
Brevibacterium	0	0	0	0	0
Enterobacter	7	0	4	2	0
Flavobacterium	0	1	0	0	0
Micrococcus	0	1	1	0	0
Proteus	0	0	0	0	0
Pseudomonas	0	0	0	2	0
Serratia	0	8	1	2	0
Staphylococcus	0	0	0	1	0
Yeast	7	10	10	7	6
Hyphal Fungi	0	0	0	0	0
TOTAL	21	30	22	20	7

TABLE IV (continued)

	July Gallery Position				
Genus	Head	Middle	Egg Niche	Larval Gallery	Frass
Achromobacter*	2	12	10	5	0
Alcaligenes	1	0	3	2	0
Aeromonas	2	0	0	1	0
Bacillus	2	2	0	0	0
Brevibacterium	1	1	0	1	0
Enterobacter	6	5	3	1	0
Flavobacterium	0	0	2	0	0
Micrococcus	0	1	0	0	0
Proteus	0	0	0	0	0
Pseudomonas	0	0	0	0	0
Serratia	0	1	0	0	0
Staphylococcus	0	1	1	0	0
Yeast	15	10	7	8	2
Hyphal Fungi	0	0	0	0	0
TOTAL	29	33	26	19	2

TABLE IV (continued)

	November Gallery Position				
Genus	Head	Middle	Egg Niche	Larval Gallery	Frass
Achromobacter*	2	6	3	4	2
Alcaligenes	0	6	1	1	0
Aeromonas	1	0	3	0	0
Bacillus	0	0	1	0	0
Brevibacterium	0	0	1	0	0
Enterobacter	2	3	4	5	0
Flavobacterium	3	0	1	2	0
Micrococcus	0	0	0	1	0
Proteus	0	1	0	0	0
Pseudomonas	0	1	0	1	0
Serratia	6	4	4	5	0
Staphylococcus	0	0	0	1	0
Yeast	5	7	11	5	1
Hyphal Fungi	0	0	0	0	0
TOTAL	19	28	30	25	3

TABLE IV (continued)

	December Gallery Position				
Genus	Head	Middle	Egg Niche	Larval Gallery	Frass
Achromobacter*	2	2	0	2	0
Alcaligenes	3	0	0	1	0
Aeromonas	2	0	1	1	0
Bacillus	0	1	0	1	0
Brevibacterium	0	0	0	0	0
Enterobacter	2	2	1	3	0
Flavobacterium	4	2	1	0	0
Micrococcus	1	1	0	0	0
Proteus	1	0	1	0	0
Pseudomonas	2	1	0	0	0
Serratia	2	3	6	3	0
Staphylococcus	0	4	0	0	0
Yeast	9	14	13	12	0
Hyphal Fungi	0	0	0	2	0
TOTAL	28	30	23	25	0

TABLE IV (continued)

	January Gallery Position				
Genus	Head	Middle	Egg Niche	Larval Gallery	Frass
Achromobacter*	1	2	1	3	0
Alcaligenes	4	0	1	3	0
Aeromonas	0	0	2	1	0
Bacillus	2	3	1	2	0
Brevibacterium	0	0	0	0	0
Enterobacter	3	4	6	7	0
Flavobacterium	1	1	0	1	0
Micrococcus	2	1	2	1	0
Proteus	0	0	0	1	0
Pseudomonas	1	0	1	0	0
Serratia	0	3	3	1	0
Staphylococcus	0	2	1	0	0
Yeast	11	8	6	6	1
Hyphal Fungi	0	0	0	0	0
TOTAL	25	24	24	26	1

*_Achromobacter_ has been dropped in form of _Acinetobacter_ in the 8th edition of Bergey's manual.

TABLE V

PERCENTAGE FREQUENCY OF GENERA ISOLATED EACH MONTH AND POSITION

Gallery Position	Mo.	Achr.	Alcal.	Aer.	Bac.	Brevi.	Ent.	Flav.	Micr.	Prot.	Pseud.	Serr.	Staph.	Yeast	Hyph. Fungi	TOTAL%
1	June	14.3	19.0	0	0	0	33.3	0	0	0	0	0	0	33.3	0	99.9
	July	6.9	3.4	6.9	6.9	3.4	20.7	0	0	0	0	0	0	51.7	0	99.9
	Nov.	10.5	0	5.3	0	0	10.5	15.8	0	0	0	31.6	0	26.3	0	100
	Dec.	7.1	10.8	7.1	0	0	7.1	14.3	3.6	3.6	7.1	7.1	0	32.2	0	100
	Jan.	4.5	16.7	0	8.0	0	1 2	4	8.0	0	4.8	0	0	44	0	100
2	June	16.7	10.0	0	6.7	0	0	3.3	3.3	0	0	26.7	0	33.3	0	100
	July	36.4	0	0	6.1	3.0	15.2	0	3.0	0	0	3.0	3.0	30.3	0	100
	Nov.	21.4	21.4	0	0	0	10.7	0	0	3.6	3.6	14.3	0	25.0	0	100
	Dec.	6.7	0	0	3.3	0	6.7	6.7	3.3	0	3.3	10.0	13.3	46.7	0	100
	Jan.	8.3	0	0	12.5	0	16.7	4.2	4.2	0	0	12.5	8.3	33.3	0	100
3	June.	18.2	0	0	0	9.1	18.2	0	4.5	0	0	4.5	0	45.5	0	100
	July	38.5	11.5	0	0	0	11.5	7.7	0	0	0	0	3.8	26.9	0	99.9
	Nov.	10.0	3.3	10.0	3.3	3.3	13.3	3.3	0	0	0	13.3	0	36.8	3.3	99.9
	Dec.	0	0	4.3	0	0	4.3	4.3	0	4.3	0	26.2	0	56.5	0	99.9
	Jan.	4.2	4.2	8.3	4.2	.0	25.0	.0	8.3	0	4.2	12.5	4.2	25.0	0	100.1
4	Jan.	20.0	10.0	0	0	0	10.0	0	0	0	10.0	10.0	5.0	35.0	0	100
	July	26.3	10.5	5.3	0	5.3	10.5	0	0	0	0	0	0	42.1	0	100
	Nov.	16.0	4.0	0	0	0	20.0	8.0	4.0	0	4.0	20.0	4.0	20.0	0	100
	Dec.	8.0	4.0	4.0	4.0	0	12.0	0	0	0	0	12.0	0	48.0	8.0	100
	Jan.	11.5	11.5	3.8	7.7	0	26.9	3.8	3.8	3.8	0	3.8	0	23.2	0	99.8
5	June	16.7	0	0	0	0	0	0	0	0	0	0	0	83.3	0	100
	July	0	0	0	0	0	0	0	0	0	0	0	0	100	0	100
	Nov.	66.7	0	0	0	0	0	0	0	0	0	0	0	33.3	0	100
	Dec.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Jan.	0	0	0	0	0	0	0	0	0	0	0	0	100	0	100

TABLE VI

THE PERCENTAGE FREQUENCY OF GENERA ISOLATES BY POSITION AND MONTH

Gallery Position	Mo.	Achr.	Alcal.	Aer.	Bac.	Brevi.	Ent.	Flav.	Micr.	Prot.	Pseud.	Serr.	Staph.	Yeast	Fungi
1	June	3.9	11.4	0	0	0	9.8	0	0	0	0	0	0	3.7	0
	July	2.5	2.9	14.3	11.8	16.7	8.4	0	0	0	0	0	0	7.9	0
	Nov.	2.5	0	7.1	0	0	2.8	15.8	0	0	0	11.5	0	2.6	0
	Dec.	2.5	8.6	14.3	0	0	2.8	21.0	8.3	25.0	22.2	3.8	0	4.7	0
	Jan.	1.2	11.4	0	11.8	0	4.2	5.3	16.6	0	11.1	0	0	5.8	0
2	June	6.5	8.6	0	11.8	0	0	5.3	8.3	0	0	15.4	0	5.3	0
	July	15.7	0	0	11.8	16.7	7.0	0	8.3	0	0	1.9	9.1	5.3	0
	Nov.	7.8	17.1	0	0	0	4.2	0	0	25.0	11.1	7.7	0	3.7	0
	Dec.	2.5	0	0	5.9	0	2.8	10.5	8.3	0	11.1	5.8	36.4	7.4	0
	Jan.	2.5	0	0	17.6	0	5.6	5.3	8.3	0	0	5.8	18.2	4.2	0
3	June	5.2	0	0	0	33.3	5.6	0	8.3	0	0	1.9	0	5.3	0
	July	13.1	8.6	0	0	0	4.2	10.5	0	0	0	0	9.1	3.7	0
	Nov.	3.8	2.9	21.4	5.9	16.7	5.6	5.3	0	0	0	7.7	0	5.8	0
	Dec.	2.5	0	7.1	0	0	1.5	5.3	0	25.0	0	11.5	0	6.8	0
	Jan.	1.2	2.9	14.3	5.9	0	8.4	0	16.6	0	11.1	5.8	9.1	3.2	0
4	June	5.2	5.7	0	0	0	2.8	0	0	0	22.2	3.8	9.1	3.7	0
	July	8.5	5.7	7.1	0	16.7	2.8	0	0	0	0	0	0	4.2	0
	Nov.	5.2	2.9	0	0	0	7.0	10.5	8.3	0	11.1	9.6	9.1	2.6	0
	Dec.	2.5	2.9	7.1	5.9	0	4.2	0	0	0	0	5.8	0	6.3	100
	Jan.	3.8	8.6	7.1	11.8	0	9.8	5.3	8.3	25.0	0	1.9	0	3.2	0
5	June	1.2	0	0	0	0	0	0	0	0	0	0	0	2.6	0
	July	0	0	0	0	0	0	0	0	0	0	0	0	1.1	0
	Nov.	2.5	0	0	0	0	0	0	0	0	0	0	0	0.5	0
	Dec.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Jan.	0	0	0	0	0	0	0	0	0	0	0	0	0.5	0
TOTAL %		100.2	99.9	99.8	100.2	100.1	99.8	100.1	99.8	100	99.9	100	100.1	100.1	100

LITERATURE CITED

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